

Characterization of cDNA clones of the family of trypsin/ α -amylase inhibitors (CM-proteins) in barley (*Hordeum vulgare* L.)

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Summary. Recombinants encoding members of the trypsin/ α -amylase inhibitors family (also designated CM-proteins) were selected from a cDNA library prepared from developing barley endosperm. Inserts in two of the clones, pUP-13 and pUP-38, were sequenced and found to encode proteins which clearly belong to this family, as judged from the extensive homology of the deduced sequences with that of the barley trypsin inhibitor CMe, the only member of the group for which a complete amino acid sequence has been obtained by direct protein sequencing. These results, together with previously obtained N-terminal sequences of purified CM-proteins, imply that there are at least six different members of this dispersed gene family in barley. The relationship of this protein family to the B-3 hordein and to reserve prolamins from related species is discussed in terms of their genome structure and evolution.

Key words: Barley endosperm – Trypsin/ α -amylase inhibitors – CM-proteins – cDNA clones

Introduction

The albumins and globulins of cereal endosperm are made up of over 20 major and many minor components, as can be shown by two-dimensional electrophoresis or by high-performance liquid chromatography (see García-Olmedo et al. 1984; Lázaro et al. 1985).

It is becoming evident that a substantial fraction of these proteins form a homologous group, encoded by a multi-gene family which is dispersed over several chromosomes (García-Olmedo and Carbonero 1970; Aragoncillo et al. 1975; Paz-Ares et al. 1983a; Salcedo et al. 1984; García-Olmedo et al. 1984; Shewry et al. 1984; Lázaro et al. 1985; Sanchez-Monge et al. 1986; Barber et al. 1986). This group of proteins, which can be extracted by chloroform/methanol mixtures (CM-proteins) and which is also soluble in aqueous alcohols, includes inhibitors of trypsin and α -amylases of diverse origin, as well as proteins with no known *in vitro* activity (Shewry et al. 1984; Barber et al. 1986). It has been speculated that this group of proteins could be involved in the protection of the endosperm tissue and might be relevant in relation to its nutritive value (Buonocore et al. 1977).

In barley (*Hordeum vulgare* L.), one α -amylase and two trypsin inhibitors, and two CM-proteins of undefined activity, have been shown to be homologous on the basis of their N-terminal sequences and overall amino acid compositions (Barber et al. 1986). We report here the characterization of cDNA clones that correspond to members of this multi-gene family.

Materials and methods

Plant material

Hordeum vulgare cv. 'Bomi' was the gift of H. Doll (Risø National Laboratory, Denmark). Developing endosperms were collected at approximately 20 days post-anthesis, by mechanical extrusion into liquid nitrogen using the method of O'Dell and Thompson (1982).

Reagents

Oligo (dT)-cellulose was obtained from Collaborative Research. AMV-reverse transcriptase was from Life Sciences Inc. Terminal transferase and S_1 -nuclease were obtained from P-L Biochemicals. Restriction endonucleases and other enzymes were supplied by Boehringer, Amersham, New England Biolabs or Bethesda Research Laboratories. [35 S]-cysteine, and

[α - 32 P] dATP and [γ - 32 P] ATP were from New England Nuclear and Amersham, respectively.

RNA extraction and in vitro protein synthesis

Total polysomal RNA and membrane-bound polysomal RNA were prepared from endosperm tissue ground to a fine powder under liquid nitrogen, as described by Paz-Ares et al. (1983 b). The poly(A)⁺RNA fraction, isolated by chromatography on oligo-dT-cellulose, was translated in a wheat germ cell-free system prepared by us from commercial wheat germ (General Mills, USA).

Size fractionation of CH₃HgOH-denatured RNA was carried out by sucrose gradient centrifugation. Gradient fractions were analysed by in vitro translation, using [35 S]cysteine, followed by SDS-polyacrylamide gel electrophoresis of total translation products and fluorography, as previously reported (Paz-Ares et al. 1983 b).

Construction of a cDNA library

The construction of the cDNA library was essentially as described by Maniatis et al. (1982). Poly(A)⁺RNA obtained from developing barley endosperm was used as a template for the synthesis of a complementary DNA strand (cDNA). Double stranded DNA (ds-cDNA) was synthesized with the Klenow fragment of DNA-poll. After S₁-nuclease treatment, the ds-cDNA was size-fractionated by preparative electrophoresis in low-melting agarose, and the fraction of molecular weight higher than 350 bp was purified by the method of Langridge et al. (1980). This fraction was used for cloning in the *Pst*I site of plasmid pBR322 after homopolymeric tailing (poly G/poly C). Cells of *Escherichia coli* strain Mc1061 made competent by the CaCl₂ procedure, were transformed with the recombinant plasmid and selected for tetracycline resistance. About 10⁴ colonies obtained from approximately 300 ng of ds-cDNA were sensitive to ampicillin and resistant to tetracycline (Amp^rTet^r).

Screening of the cDNA library

The Amp^rTet^r bacterial colonies were further screened in two steps. In the first step, colony hybridization was carried out according to the Grunstein and Hogness (1975) procedure. Probes were prepared by synthesis of radioactive cDNA with the AMV reverse transcriptase, from appropriate RNA gradient fractions. In the second step, two rounds of hybrid-release translation were performed with the clones selected in the first step.

Hybrid-selected translation

Pools of five clones were grown in LB medium and their plasmids purified according to Birnboim and Doly (1979). Plasmid DNA was then covalently bound to diazobenzyl-oxymethyl (DBM) paper (Christophe et al. 1982). The hybridization buffer was: 50% formamide, 800 mM NaCl, 100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.4% SDS, 2 mg/ml yeast tRNA. The filters were preincubated in this buffer for 60 min at 37 °C. After incubation with total polysomal RNA (100–500 µg/ml) in the same buffer for the same period of time, the filters were washed with 150 mM NaCl and then the selected mRNA was finally released with buffer without salt, heating for 5 min at 100 °C and rapidly cooling at 0 °C. In vitro protein synthesis was carried out as indicated above.

DNA sequencing

The method of Maxam and Gilbert (1980) was used for DNA sequencing of subclones obtained in plasmids pUC-12 or

pUC-13 (Messing 1983). Restriction maps were carried out by standard techniques (Maniatis et al. 1982).

Results

A cDNA library from developing endosperm was constructed in the pBR322/*E. coli* Mc1061 vector system using poly(A)⁺RNA from total polysomal RNA as starting template. A preliminary screening of this library for recombinants corresponding to the CM-proteins family was carried out with a radioactive cDNA probe. This probe was prepared from a gradient fraction that was enriched in mRNA for these proteins, as described by Paz-Ares et al. (1983 b). About 20% of the colonies gave both a positive signal with this probe and a negative one with a second probe which was similarly prepared from a pool of RNA fractions of higher sedimentation velocity (mRNA's for B- and C-hordeins and ribosomal RNA). Recombinants selected in this preliminary screening were further tested by hybrid-selected translation, electrophoresis and fluorography, first in pools of five clones and then as individual ones, as shown in Fig. 1. Clones pUP-13 and pUP-38 selected mRNA's that encoded proteins which were efficiently

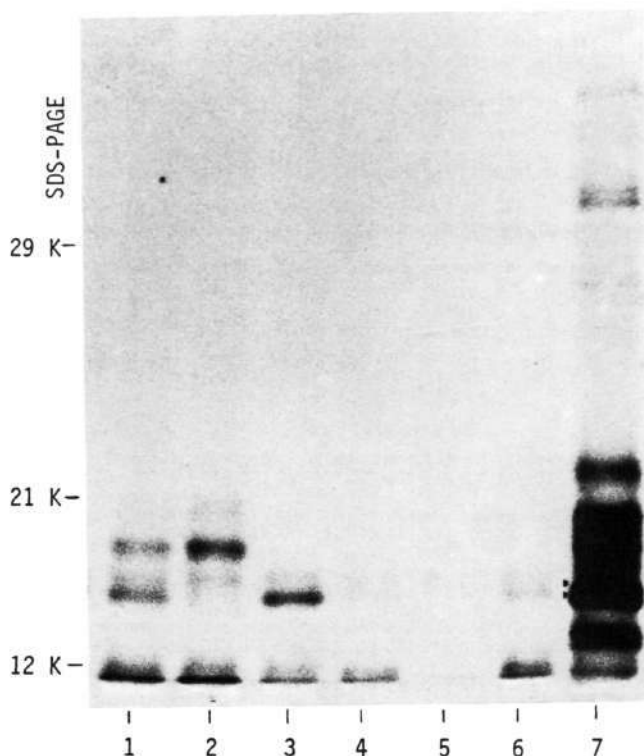


Fig. 1. Hybrid-release translation of messengers selected by a pool of five clones (1) and the individual clones from the same pool (2–6). Lanes 2 and 3 correspond to clones pUP-38 and pUP-13, respectively (7). Translation products from total poly (A)⁺RNA. [35 S] cysteine was used as precursor in all cases

The figure consists of two schematic diagrams of DNA sequences. The top diagram represents the genomic organization of the PUP-38 gene. It shows a horizontal line with several restriction sites marked: AluI, HaeIII, SmaI, BamHI, and Sau3a. A scale bar indicates 100 bp. Below this, two arrows indicate the direction of transcription for the 5' and 3' transcripts. The bottom diagram shows the structure of the pUP-38 plasmid. It shows a horizontal line with restriction sites marked: AluI, SmaI, HaeIII, and SmaI. Two arrows indicate the direction of transcription for the 5' and 3' transcripts.

labeled with [³⁵S]cysteine, had apparent sizes within the range observed for the precursors of CM-proteins, and gave a weak antigenic reaction with monospecific antibodies raised against protein CMd (not shown). The inserts in these two clones were sequenced according to Maxam and Gilbert (1980), following the sequencing strategies outlined in Fig. 2. The nucleotide sequences are presented together with the amino acid sequences deduced from their longest reading frames in Fig. 3. The cDNA in clone pUP-13 encodes a sequence of 136 amino acids in which, following the criteria of Heijne (1983), the hydrophobic core and the cleavage site (after position 13) of a signal peptide can be discerned in the N-terminal domain, leaving a mature protein of 123 amino acids. This is in agreement with previous evidence from experiments of *in vivo* and *in vitro* synthesis, which indicated that CM-proteins were synthesized by membrane-bound polysomes as larger precursors that seemed to be co-translationally processed (Paz-Ares et al. 1983 b). The cDNA in clone pUP-38 does not encode the N-terminal end of the corre-

pUP-38

	leu pro glu trp met thr ser ala glu leu asn tyr pro gly gln pro tyr leu ala lys	20
1	TTA CCC GAA TGG ATG ACA TCC GCG GAG CTG AAC TAC CCC GGG CAG CCA TAC CTC GCC AAG	
	leu tyr cys cys gln glu leu ala glu ile pro gln gln cys arg cys glu ala leu arg	40
61	TTG TAT TGT TGC CAA GAG CTT GCA GAA ATT CCC CAG CAG TGC CGG TGC GAG GCG CTG CCG	
	thr ser met ala leu pro val pro pro gln pro val asp pro ser thr gly asn val gly	60
121	ACT TCA ATG GCG TTG CCG GTA CCG CCT CAG CCC GTG GAC CCG AGC ACC GGC AAT GTT GGT	
	gln ser gly leu met asp leu pro gly cys arg glu met gln arg asp phe val arg	80
181	CAG AGC GGC CTC ATG GAG CTG CCC GGA TGC CCC AGG GAG ATG CAA CGG GAC TTC GTC AGA	
	leu leu val ala pro gly gln cys asn leu ala thr ile his asn val arg tyr cys pro	100
241	TTA CTC GTC GCC CCG GGG CAG TGC AAC TTG GCG ACC ATT CAC AAC GTT CGA TAC TGC CCC	
	ala val glu gln pro leu trp ile * * * *	108
301	GCC GTG GAA CAG CCG CTG TGG ATC TAG TGA TGA TAA AATCAGTCGTTCTGTAATAAGCATGC	
	1 2 3 4 5	
363	ATGTTGCGTACATAGGCGTAGGCGTGTGCGTGTGGTGTGCATGTATGCATATGTGAGCTCCGCACG	
	poly A	
429	CTCAACATGTGTGGGCTATCTGCTATGAACGAGATAAAGAGAACCATTTTGTGGTTCTTTAATT	
495	CA ₃₂	

Fig. 3. Nucleotide sequences and deduced amino acid sequences of clones pUP-13 and pUP-38. The vertical arrow (∇) indicates the site of cleavage of the leader sequence deduced according to Heijne (1983). Segments numbered 1–5 indicate homologies in the non-coding 3'-ends. Horizontal arrows indicate repeats. Poly A indicates polyadenylation signals. The base marked with a dot (•) has not been corroborated in both strands. Stop signals are indicated with asterisks (*)

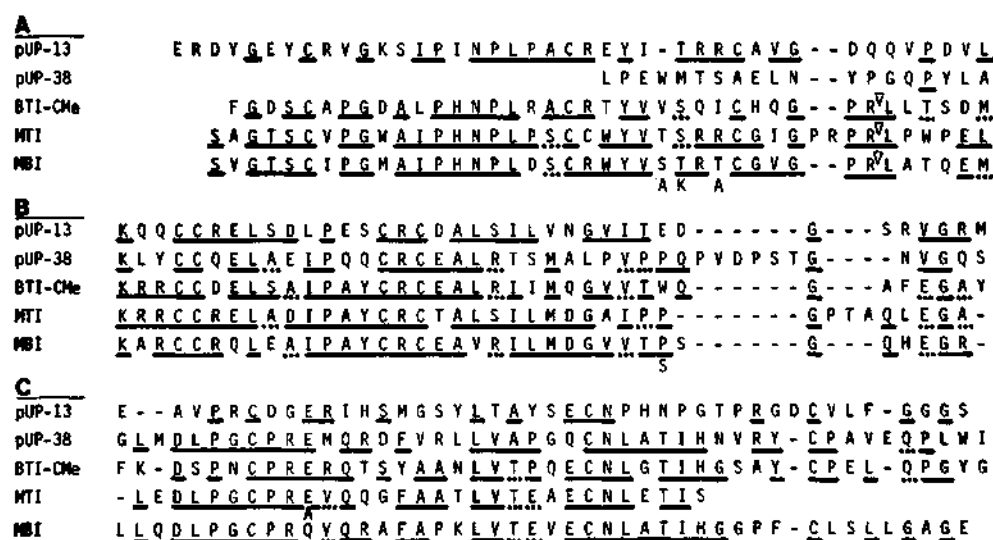


Fig. 4. Alignment of amino acid sequences deduced from clones pUP-13 and pUP-38 with those of barley trypsin inhibitor CMe (BTI-CMe; Odani et al. 1983), maize trypsin inhibitor (MTI; Mahoney et al. 1984) and millet bifunctional inhibitor (MBI; Campos and Richardson 1983). Gaps have been introduced to optimize alignment. Homologies have been underlined (— or ...). The sequences have been divided into the A, B, and C domains defined by Kreis et al. (1985)

Discussion

A weak homology has been reported by Kreis et al. (1985) between the A, B, and C domains of trypsin inhibitor CMe and α -gliadin and γ -secalin. It is of interest to note that the A and B domains of the pUP-13 protein and the B domain of that encoded by pUP-38 are closer than CMe to the above-mentioned reserve proteins. Furthermore, the A domain of pUP-13 is significantly closer to the corresponding region of the B-3 hordein reported by Forde et al. (1985) than to the same regions of α -gliadin and γ -secalin (sequences not shown). The homologies of the C domains of the proteins in Fig. 4 with respect to the above-mentioned reserve proteins are very weak in all cases. Genes encoding CM-proteins in barley have been found in chromosomes 1, 3, and 4 (Salcedo et al. 1984), so the relationship to the B-3 hordein (chromosome 5) implies that the ancestral sequence must have been dispersed over at least four of the seven barley chromosomes.

We have previously presented evidence of considerable divergence within this family at the levels of structure, in vitro activity and genetic regulation (Lázaro et al. 1985; Barber et al. 1985). A comparison of the 3'-end, non-coding regions of the two clones shows that although some homology has been conserved, sequence divergence is considerable and some relevant features, such as the invert repeats, are not shared.

Acknowledgements. We would like to thank Drs. C. Escarmis and A. González for useful advice with the DNA sequencing, and C. Rojas and L. Lamóneda for technical assistance. Financial support from the Comisión Asesora de Investigación Científica y Técnica (grant no. 1344) is also acknowledged.

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